Supplementary materials

Antibodies used

The following antibodies were used: PerCP conjugated CD3 (clone SP34-2; BD Biosciences), FITC conjugated CCR7 (clone 150503; R&D systems), PE conjugated α4β7 (clone Act 1; National Institutes of Health Non-human Primate Reagent Resource), PerCP conjugated HLA-DR (clone G46-6; BD Biosciences), APC conjugated CD45RO (clone UCHL1; Tonbo), PE-Cy7 conjugated CCR5 (clone 2D7;BD Biosciences), PE-TxRd conjugated CD38 (clone HIT2; MHCD), V450 conjugated CD45 (clone HI30; Tonbo), V500 conjugated CD8 (clone SK1; BD Biosciences), BV650 conjugated CD4 (clone OKT4; Biolegend), Alexa700 conjugated Ki-67 (clone B56; BD Biosciences), FITC conjugated IL-17A (Clone eBio64Dec15; ebiosciences), Pacific Blue conjugated IFNγ (Clone B27; BD Biosciences), and Alexa700 conjugated TNFα (clone MAb11; BD Biosciences).

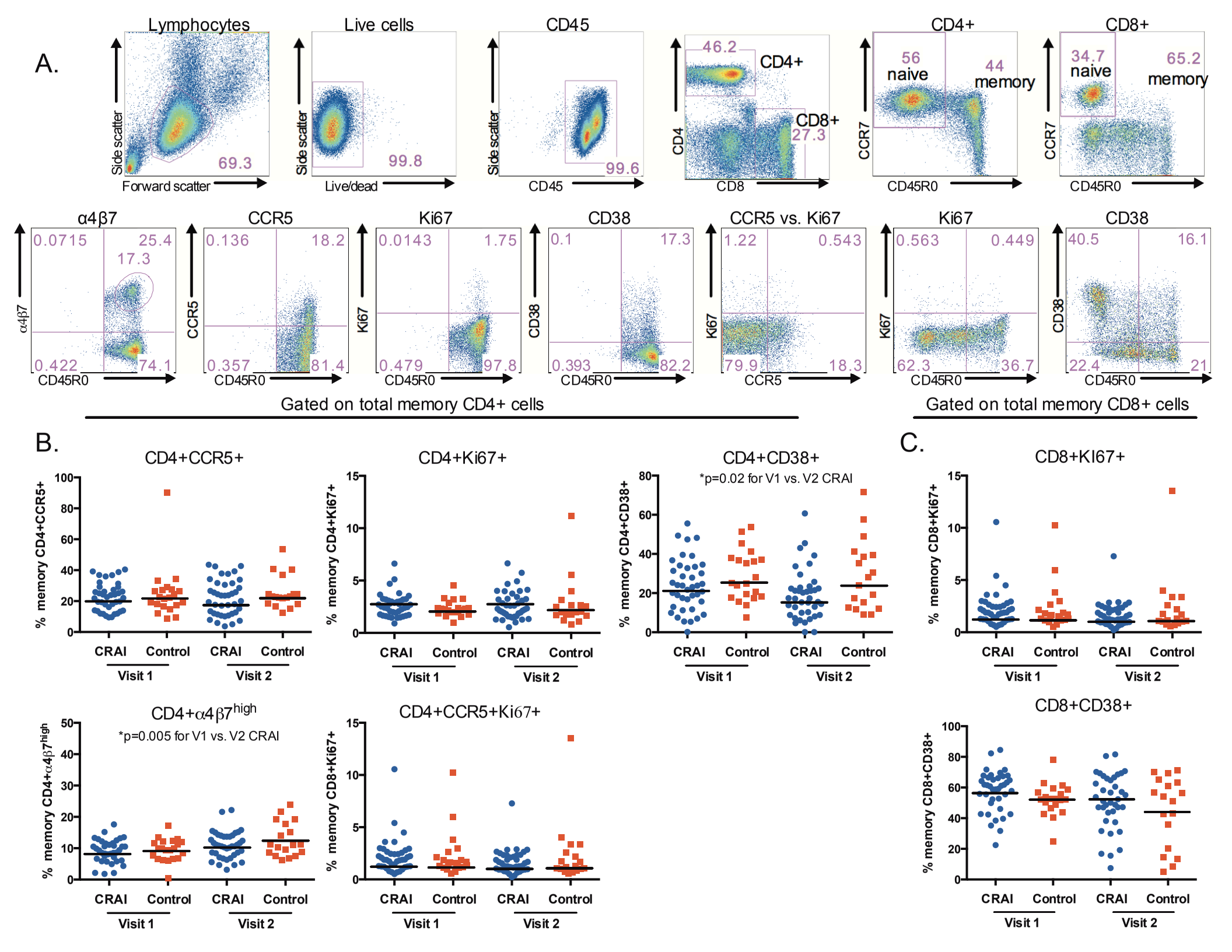
Supplementary Table 1. Modeling results for blood PBMC phenotyping and functional analyses. Results of repeated measured modeling analysis for blood PBMC phenotyping and intracellular cytokine staining after mitogen stimulation for MSM engaging in CRAI (CRAI) and men who never engaged in anal intercourse (controls). MSM abstained from CRAI for ≥72 hours prior to visit 1 and engaged in CRAI ≤24 hours prior to visit 2. Mixed effects models controlled for time, time by group interactions, for the laboratory technician performing the assay, age, and race.

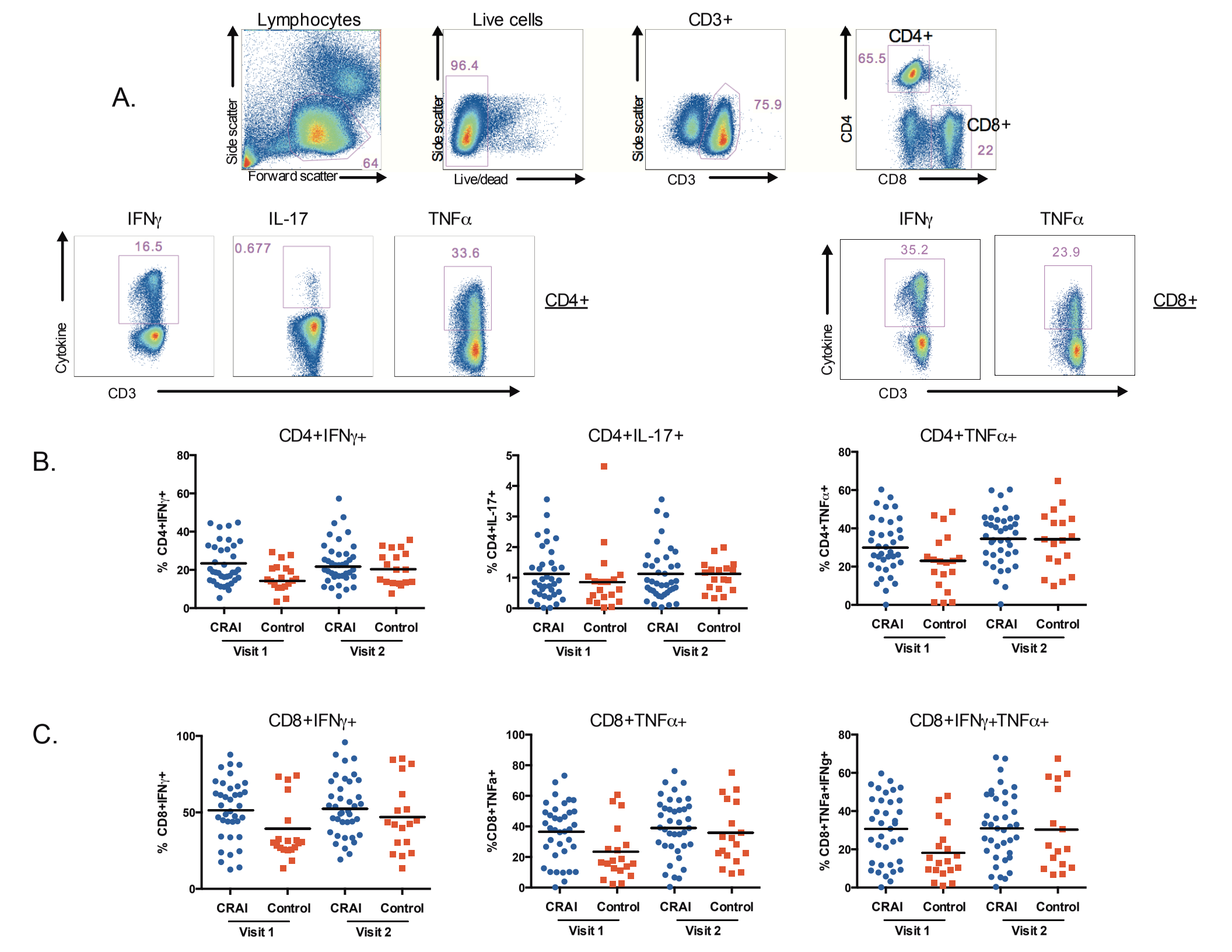
Immunologic Index	n	Visit 1 mean (95% CI)	n	Visit 2 mean (95%CI)	adjusted model-based mean	p-value	CRAI: V1 vs. V2 Mean difference or ratio**	p-value
Blood Memory CD4+ cells								
*%CCR5+								
CRAI	40	20.8(18.2,23.8)	40	18.6(15.2,22.7)	19.7(17.0,22.7)		1.1**	0.2
Control	21	22.1(18.3,26.6)	18	24.8(20.9,29.4)	23.4(20.1,27.3)	0.1	n/a	
*%Ki67+								
CRAI	40	2.5(2.2,2.8)	40	2.4(2.0,2.8)	2.4(2.2,2.7)		1.0**	0.7
Control	21	2.2(1.9,2.5)	18	2.2(1.7,2.9)	2.2(1.9,2.6)	0.3	n/a	
*%CD38								
CRAI	39	21.2(17.3,25.9)	38	17.3(14.1,21.1)	19.1(16.0,22.9)		**1.2****	**0.02**
Control	21	25.8(21.4,31.2)	18	24.5(18.8,31.8)	25.2(20.4,31.0)	0.07	n/a	
*%CCR5+Ki67+								
CRAI	40	1.1(0.9,1.3)	40	1.0(0.8,1.2)	1.0(0.9,1.2)		1.2**	0.3
Control	21	1.0(0.8,1.3)	18	1.0(0.7,1.4)	1.0(0.8,1.3)	0.9	n/a	
%$\alpha4\beta7^{high}$+								
CRAI	40	8.5(7.1,9.8)	40	10.8(9.5,12.1)	9.6 (8.6,10.7)		**-2.3**	**0.005**
Control	21	9.7(8.3,11.1)	18	12.7(10.3,15.1)	11.2(9.5,12.9)	0.1	n/a	
Blood Memory CD8+ cells								
*%Ki67+								
CRAI	40	1.9(1.5,2.2)	40	1.5(1.2,1.8)	1.7(1.5,1.9)		1.3**	0.10
Control	21	1.6(1.2,2.1)	18	1.6(1.1,2.2)	1.6(1.3,2.0)	0.8	n/a	
%CD38+								
CRAI	39	57.6(52.9,62.2)	38	51.6(45.9,57.2)	54.6(50.5,58.6)		6.0	0.06
Control	21	51.4(47.8,55.0)	18	44.5(35.2,53.8)	48.0(42.9,53.0)	0.05	n/a	
Stimulated blood CD4+ cells								
%IFNγ+								
CRAI	37	22.6(19.0,26.1)	39	23.5(20.2,26.9)	23.0(20.2,25.9)		-1.0	0.6
Control	19	16.3(12.7,19.8)	18	21.9(17.7,26.0)	19.1(15.7,22.4)	0.08	n/a	
%TNFα+								
CRAI	37	30.6(25.6,35.6)	39	34.8(30.4,39.3)	32.7(29.1,36.3)		-4.2	0.2
Control	19	21.7(14.3,29.0)	18	34.0(26.3,41.7)	27.8(21.4,34.3)	0.2	n/a	
%IL-17+								
CRAI	37	1.1(0.8,1.4)	39	1.1(0.8,1.3)	1.1(0.9,1.3)		0.01	0.9
Control	19	0.9(0.4,1.4)	18	1.0(0.8,1.3)	1.0(0.7,1.3)	0.6	n/a	
Stimulated blood CD8+ cells								
%IFNγ+								
CRAI	37	49.9(44.0,55.9)	39	51.5(45.6,57.3)	50.7(45.6,55.8)		-1.6	0.6
Control	19	42.3(33.2,51.4)	18	50.5(40.0,61.0)	46.4(37.3,55.5)	0.4	n/a	
%TNFα+								
CRAI	37	35.2(28.9,41.5)	39	38.5(32.5,44.5)	36.8(31.9,41.8)		-3.3	0.4
Control	19	25.6(16.7,34.5)	18	37.1(27.3,46.9)	31.4(22.9,39.9)	0.3	n/a	
% IFNγ+TNFα+								
CRAI	37	29.4(23.5,35.3)	39	30.6(25.1,36.1)	30.0(25.3,34.7)		-1.2	0.7
Control	18	20.7(13.6,27.8)	16	32.0(22.1,41.8)	26.3(18.6,34.1)	0.4	n/a	

* report the geometric mean (95%CI) **ratio of visit 1 and 2 geometric means reported CRAI=condomless receptive anal intercourse.

Supplementary Figure 1. Blood PBMC phenotyping shows no increase in HIV target cells among MSM engaging in CRAI. (A) Representative gating strategy for blood PBMCs. Lymphocytes were identified by forward and side scatter, then live cells were identified by live/dead staining. CD45+ cells were then separated into CD4+ and CD8+ subsets. Memory CD4+ and CD8+ cells were identified by excluding CCR7+CD45RO- cells. Memory CD4+ cells were then assessed for expression of α4β7 (gut homing and HIV co-receptor), CCR5 (HIV co-receptor), Ki67 (proliferation), CD38 (activation), and co-expression of CCR5 and Ki67. Memory CD8+ cells were assessed for expression of Ki67 (proliferation) and CD38 (activation). (B) Results of CD4+ cell phenotyping for MSM engaging in CRAI and controls at visit 1 (abstained from CRAI for ≥ 72 hours for MSM engaging in CRAI) and visit 2 (MSM engaged in CRAI ≤ 24 hours prior). Black lines represent visit 1 and visit 2 model-based means as reported in Table 2. (C) Results of CD8+ cell phenotyping for MSM engaging in CRAI and controls at visit 1 and visit 2. Black lines represent visit 1 and visit 2 model-based means as reported in Supplementary Table 1.

Supplementary Figure 2. Cytokine production upon mitogen stimulation of CD4+ and CD8+ blood PBMCs shows minimal differences between MSM engaging in CRAI and controls. Representative gating strategy for blood PBMcs after stimulation with PMA/Ionomycin (A). Lymphocytes were identified by forward and side scatter, then live cells were identified by live/dead staining. CD3+ cells were then separated into CD4+ and CD8+ subsets. Unstimulated and stimulated CD4+ and CD8+ cells were assessed for IFNγ, IL-17, and TNFα production. (B) Results of CD4+ cell cytokine production for MSM engaging in CRAI and controls at visit 1 (abstained from CRAI for ≥ 72 hours for MSM engaging in CRAI) and visit 2 (MSM engaged in CRAI ≤ 24 hours prior). Black lines represent visit 1 and visit 2 model-based means as reported in Table 2. (C) Results of CD8+ cell cytokine production for MSM engaging in CRAI and controls at visit 1 and visit 2. Black lines represent visit 1 and visit 2 model-based means as reported in Supplementary Table.


A.
Lymphocytes
Side scatter
Forward scatter
69.3
Live cells
Side scatter
Live/dead
99.8
CD45
Side scatter
CD45
99.6
46.2
CD4+
CD8+
27.3
CD4
CD8
CD4+
56
naive
44
memory
CCR7
CD45R0
CD8+
34.7
naive
65.2
memory
CCR7
CD45R0
α4β7
0.0715
25.4
17.3
0.422
74.1
α4β7
CD45R0
CCR5
0.136
18.2
0.357
81.4
CCR5
CD45R0
Ki67
0.0143
1.75
0.479
97.8
Ki67
CD45R0
CD38
0.1
17.3
0.393
82.2
CD38
CD45R0
CCR5 vs. Ki67
1.22
0.543
79.9
18.3
Ki67
CCR5
Ki67
0.563
0.449
62.3
36.7
Ki67
CD45R0
CD38
40.5
16.1
22.4
21
CD38
CD45R0
Gated on total memory CD4+ cells
Gated on total memory CD8+ cells
B.
CD4+CCR5+
% memory CD4+CCR5+
CRAI
Control
CRAI
Control
Visit 1
Visit 2
CD4+Ki67+
% memory CD4+Ki67+
CRAI
Control
CRAI
Control
Visit 1
Visit 2
CD4+CD38+
*p=0.02 for V1 vs. V2 CRAI
% memory CD4+CD38+
CRAI
Control
CRAI
Control
Visit 1
Visit 2
C.
CD8+KI67+
% memory CD8+Ki67+
CRAI
Control
CRAI
Control
Visit 1
Visit 2
CD4+α4β7high
*p=0.005 for V1 vs. V2 CRAI
% memory CD4+α4β7high
CRAI
Control
CRAI
Control
Visit 1
Visit 2
CD4+CCR5+Ki67+
% memory CD8+Ki67+
CRAI
Control
CRAI
Control
Visit 1
Visit 2
CD8+CD38+
% memory CD8+CD38+
CRAI
Control
CRAI
Control
Visit 1
Visit 2


A.
Lymphocytes
Side scatter
Forward scatter
64
Live cells
96.4
Side scatter
Live/dead
CD3+
Side scatter
CD3
75.9
CD4+
65.5
CD4
CD8+
22
CD8
IFNγ
IL-17
TNFα
16.5
0.677
33.6
Cytokine
CD3
CD4+
IFNγ
TNFα
35.2
23.9
Cytokine
CD3
CD8+
B.
CD4+IFNγ+
% CD4+IFNγ+
CD4+IL-17+
% CD4+IL-17+
CD4+TNFα+
% CD4+TNFα+
CRAI
Control
CRAI
Control
Visit 1
Visit 2
C.
CD8+IFNγ+
% CD8+IFNγ+
CD8+TNFα+
%CD8+TNFa+
CD8+IFNγ+TNFα+
% CD8+TNFa+IFNg+
CRAI
Control
CRAI
Control
Visit 1
Visit 2